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PRINCIPAL INVESTIGATOR: Edward P. Gelmann, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032-3702

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Edward P. Gelmann, M.D.

E-Mail: gelmanne@columbia.edu

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14. ABSTRACT
NKX3.1 is a prostate-specific homeobox gene that maps to chromosome 8p21, the most frequent target for loss of heterozygosity in prostate cancer. NKX3.1 is a haploinsufficient tumor suppressor in the prostate. We found that IGFBP-3expreession was activated 10-fold by NKX3.1 in cell lines and tissues. IGFBP-3 is an inhibitor of IGF-1, a serum component that when elevated is a risk factor for prostate cancer. NKX3.1 expression inhibits IGFIR signaling and diminishes IRS-1 phosphorylation. Knock down of IGFBP-3 attenuates the growth suppressive effects of NKX3.1. NKX3.1 C154T is a polymorphic allele present in ~10% of the population. The polymorphic allele codes for a variant protein that replaces arginine 52 with cysteine. NXK3.1 C154T confers a minimally increased risk for prostatic enlargement and for prostate cancer. In a cohort of cases and controls with known NKX3.1 genotype we found that the effect of serum IGF-1 on prostate cancer risk was seen only in men with at least one polymorphic NKX3.1allele. Consistent with its apparent interaction with IGF-1 in prostate cancer risk, NKX3.1 R52C protein is attenuated in activation of IGFBP-3. The data therefore show that the two prostate cancer risk factors, NKX3.1 R52C and circulating IGF-1 interact. NKX3.1 R52C activates less IGFBP-3 expression than its wild type counterpart and thereby predisposes prostate epithelial cells to the proliferative and antiapoptotic effects of IGF-1, increasing the risk for prostate cancer.

15. SUBJECT TERMS
Prostate cancer, NKX3.1, IGF-1, IGFBP-3

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INTRODUCTION:

The *NKX3.1* gene is not subject to somatic mutation in prostate cancer (1, 2). Gene targeting studies in mice showed that *Nkx3.1* haploinsufficiency can predispose to prostate epithelial dysplasia and can cooperate with other oncogenic mutations to augment prostate carcinogenesis (3, 4). In gene-targeted mice haploinsufficiency of *Nkx3.1* is accompanied by decreased expression of genes under the regulation of the *Nkx3.l* homeoprotein (5). We have recently shown that diminished levels of *NKX3.1* expression in primary human prostate cancer and intraepithelial neoplasia correlated with the degree of gene inactivation by deletion, methylation, or both. There is now convincing evidence that *NKX3.1* is both a suppressor protein and is inactivated at the earliest stages of human prostate cancer. NKX3.1 binds to DNA and suppresses expression of genes downstream from cognate DNA binding sites (6). We have yet to identify a promoter that is transcriptionally activated by direct binding of NXK3.1 to its cognate DNA –TAAGTA- sequence. In fact, our experiments with reporter constructs containing NKX3.1 binding DNA suggest that NKX3.1 alone cannot initiate assembly of a transcriptional complex (6). NKX3.1 is known to interact with other transcription factors such as serum response factor (SRF) and serves as a synergistic coactivator of promoters with serum response elements such as smooth muscle γ-actin (7). We have sought to identify down stream transcription targets of NKX3.1 in order to understand the tumor suppressor properties of this transcription factor. This project is designed to characterize NKX3.1 activation of IGFBP-3, a known growth suppressor and down regulator of IGF-1 interaction with its receptor.

Task 1. Determine IGFBP-3 expression in prostate specimens from men with different NKX3.1 genotype. Months 1-18

1. *Establish baseline criteria for analysis of tissue microarrays from the Dana Farber- Harvard Prostate SPORE.*
2. *Analyze 40 specimens from Physicians Health Study, known to contain at least one NKX3.1 C154T allele for IGFBP-3, keratin, NKX3.1 and histone expression.*
3. *Analyze specimens with known NKX3.1 homozygous wild type genotype for IGFBP-3, keratin, NKX3.1 and histone expression by quantitative confocal immunomicroscopy.*

Task 1 is ongoing as it was delayed by delay in generating sections of tissue blocks from Harvard. These blocks are now in the lab at Columbia University and are being processed for NKX3.l and IGFBP-3 staining.

Task 2. Analyze serum IGF-1 and IGFBP-3 levels from a cohort of prostate cancer cases and controls that have been genotyped for NKX3.1.
Months 1-18

1. *Identify and aliquot sera from Australian case-control study of men with prostate cancer.*
2. *Batch and send samples to McGill University, Montreal, Canada.*
3. *Test samples and controls for IGF-1 with ELISA assay.*
4. *Statistical analysis of samples for correlation with NKX3.1 genotype at Columbia University.*

This task is ongoing. Samples have been sent to McGill and have been processed. Data is currently being sent back to Melbourne for compilation with *NKX3.1* genotype data and analysis will be available at the time of the next annual report.

Task 3. Determine the effect of NKX3.1 on prostate cancer cell growth and survival.

1. *Establishment of PC-3 cells expressing NKX3.1. (Months 1-12) We will establish stable PC-3 transfected cells expressing NKX3.1, NKX3.1(R52C), NKX3.1(S48A), NKX3.1(N174Q), and the expression vector pcDNA3.1.*
2. *Determination of IGFBP-3 expression in derivative PC-3 cells. (Months 6-30). We will determine relative expression of IGFBP-3 in the different PC-3 cell clones by western blotting using β-actin as a control and LNCaP cell extracts as a reference.*

3. *IGFBP-3 mRNA (Months 13-36). We will analyze expression of IGFBP-3 mRNA in stable transfectants and transiently transfected PC-3 cells by real time RT-PCR, similar to our experiments with the mouse prostate tissues, but using primers for the human cDNA. We will analyze the level of IGFBP-3 message in stably transfected clones compared to internal controls of GAPDH and β-actin cDNA.*
4. *Effect of NKX3.1 expression on PC-3 cell growth (Months 12-36). We will assay growth of PC-3 derivative cell clones by MTT and by cell counting to compare doubling times to parental and control transfected cells (with expression vector pcDNA3.1).*
5. *Determine the effect of NKX3.1 expression on PC-3 apoptosis (Months 25-36). We will assay the derivative lines for apoptosis. Apoptosis will be assayed by in-situ end labeling (ISEL) as we have previously published and by immunohistochemical assay for activated caspase-3 and cleaved PARP.*
6. *IGFBP-3 knockdown in PC-3 cells (Months 23-36). We will engineer the expression of NKX3.1 and mutants of NKX3.1 in PC-3 prostate cancer cells to determine the effect of NKX.3.1 on PC-3 cell growth and apoptosis. We will validate these results with transient transfection assays and, if necessary, with conditional expression contracts.*

I. NKX3.1 Up Regulates IGFBP-3 in Cell Lines

We observed that in an expression array of PC-3 prostate cancer cells transfected with either an empty vector or an NKX3.1 expression vector IGFBP-3 was up regulated 10-fold at the mRNA level. This was also seen by western blotting for IGFBP-3 protein (Fig 1A). To demonstrate that the activation of IGFBP-3 expression was not unique to a single transfected clone we derived multiple clones of PC-3 cells expressing NKX3.1. Each of the clones had activated IGFBP-3 protein (Fig 1B) and mRNA (Fig 1C) expression compared to a clone transfected with an empty vector containing a selectable drug marker. Furthermore, in a mass culture transient transfection of NKX3.1 into PC-3 cells also resulted in increased IGFBP-3 protein expression 48 hours after DNA transfer (Fig 1D). LNCaP prostate cancer cells that normally express NKX3.1 have adapted to the continuous expression of the suppressor protein by reducing IGFBP-3 expression. However, over expression of NKX3.1 in LNCaP cells results in increased IGFBP-3 expression as seen on western blot (Fig 1E). In comparison, another IGF-1 binding protein, IGFBP-4 did not show a similar robust over expression in PC-3 cells expressing NKX3.1 (Fig 1F).

The next step was to validate the relationship between NKX3.1 expression and IGFBP-3 expression in human tissues. We have previously described a quantitative confocal immunomicroscopic assay for NKX3.1 that can be used on human tissue samples. The assay was validated both with tissue culture cells and with murine prostate samples from *Nkx3.1* gene targeted mice (8). To apply this assay to measurement of a protein present in the cytoplasm such as IGFBP-3 we standardized the use of the IGFBP-3 and NKX3.1 antibodies on cultured cells. Quantitative immunofluorescent confocal microscopy was performed on PC-3 cells transfected with NKX3.1. Cytokeratin was used as an internal control for IGFBP-3 expression and, as we had done previously, histone was used as an internal control for NKX3.1 expression because it is a nuclear protein. Similar to our published procedure (8) we validated the confocal assay in PC-3 transfected cells (Figure 2A). We calculated a 6-fold increase in IGFBP-3 expression in PC-3(NKX3.1) cells compared to controls. Although this number differed somewhat from the quantitation by western blot densitometry, the confocal immunomicroscopic assay provided sufficient discrimination of IGFBP-3 expression to allow us to perform analysis of human tissue samples.

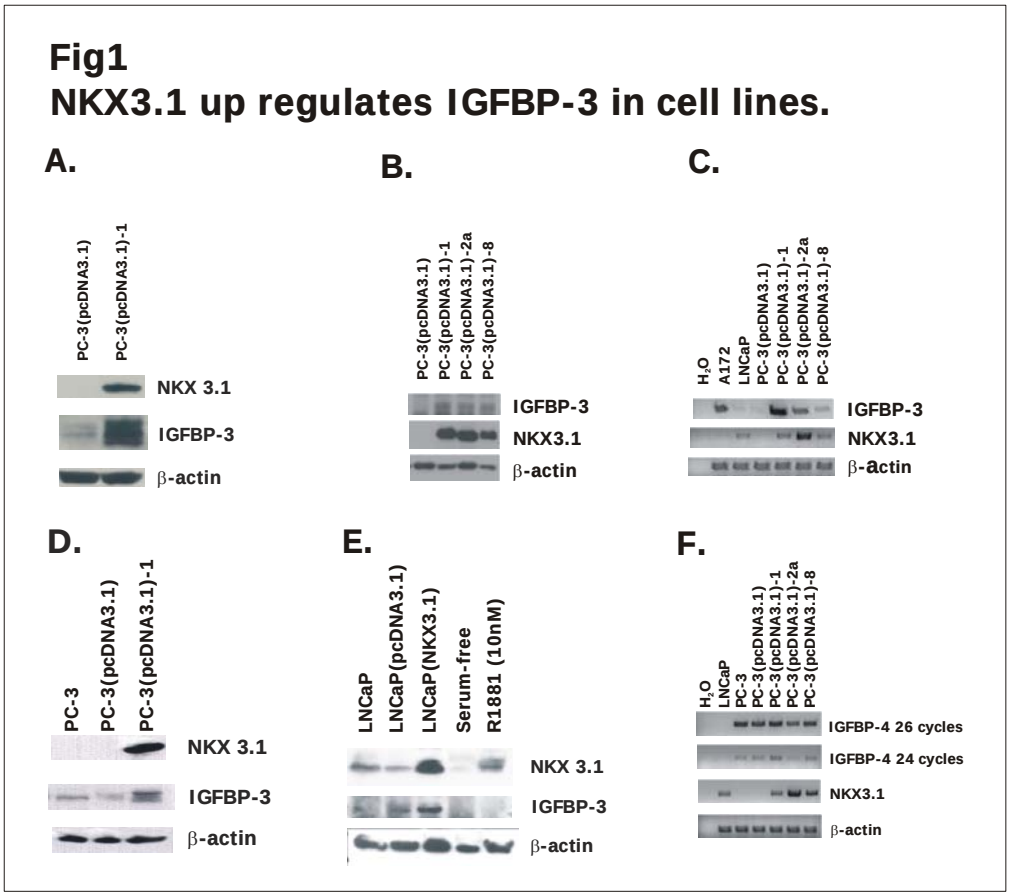


Fig 1 A. Western blot analysis of cell extracts from stable PC-3(control) and PC-3(NKX3.1) cells from the micro-array data. β-Actin is used as a loading control. B. Western blot analysis of cell extracts from stable PC-3(control) and additional PC-3(NKX3.1) cells derived after the micro-array experiment. β-Actin is used as a loading control. C. RT-PCR analysis of PC-3(control) and PC-3(NKX3.1) stable cell clones for NKX3.1 and IGFBP-3 mRNA expression. A172 cell line is a positive control for IGFBP-3 expression and LNCaP cell lines is used as a positive control for NKX3.1 expression. β-Actin is used as a loading control. D. Western blot analysis of PC-3 cell extracts that have been transiently transfected with the NKX3.1 expression vector. β-Actin is used as a loading control. E. Western blot analysis of LNCaP cell extracts that have been transiently transfected with the NKX3.1 expression vector or treated with 10nM R1881. β-Actin is used as a loading control. F. RT-PCR analysis of IGFBP-4 mRNA expression in PC-3(control) and PC-3(NKX3.1) stable cell clones. β-Actin was used as a loading control.

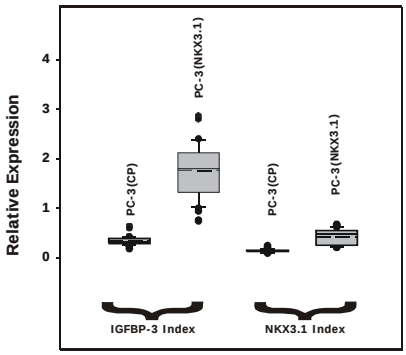
Using 27 of the 48 samples that had prior quantitation of NKX3.1 expression (8) we analyzed IGFBP-3 and cytokeratin expression in a minimum of 50 cancer and 50 nonmalignant cells from individual paraffin sections of radical prostatectomy samples. The details of the sample acquisition, analysis, and preparation are in Appendix 2. The standard errors of the individual measurements for IGFBP-3 and cytokeratin were all within 10% as we previously reported (8). Relative IGFBP-3 expression was calculated as a ratio of ratios. For each cell the ratio of IGFBP-3 expression to cytokeratin expression was determined. Then the overall mean for cancer cells was divided by the overall mean for nonmalignant cells. Thus, as with NKX3.1, each tissue section was quantitated relative to an internal control protein not expected to vary significantly based either on the malignant state of the cells nor on NKX3.1 expression. The results are shown in Figure 2B There was a good correlation (R= 0.575739) between NKX3.1 expression and IGFBP-3 expression in that when the former was reduced substantially, so was the latter. These data are consistent with the notion that NKX3.1 regulates IGFBP-3 expression in vivo. The data also demonstrate the feasibility of using this assay to evaluated IGFBP-3 expression in nonmalignant cells from men with defined *NKX3.1* genotype as proposed in Aim 1.

To obtain additional data supporting the relationship between NKX3.1 and IGFBP-3 expression we analyzed prostate mRNA in *Nkx3.1* gene targeted mice. We have published quantitation of Nkx3.1 protein in intact, *Nkx3.1*$^{+/-}$, and *Nkx3.1*$^{-/-}$mice , showing that levels of Nkx3.1 protein correlated with gene copy number in *Nkx3.1*-targeted mice [Appendix 2, (8)]. We performed real-time RT-PCR of RNA extracted from murine prostates at 4 and 12 months of age (generously provided by Cory Abate-Shen, Robert Wood Johnson School of Medicine and Dentistry, New Brunswick, NJ). The data in Figure 2C show that *Igfbp-3* mRNA expression was related to *Nkx3.1* gene copy number in murine prostates. In each reaction *Gapdh* was used as a control and was invariant between the different strains. We therefore conclude that in murine as well as human prostatic epithelial cells *Igfbp-3* expression is directly related to Nkx3.1 expression.

Fig 2. A. Analysis of confocal images of PC-3 control and PC-3(NKX3.1) cell. Confocal images were collected on an Olympus IX 70 confocal inverted microscope and analyzed using Metamorph software (Universal Imagining Corporation). Computer assisted tracing of nuclei on randomly chosen cells (n=50) from each cell type on the slide was done, while visualizing cells for Texas Red (cytokeratin and histone) staining to decrease sampling bias. After background subtraction and thresholding of the image intensity of FITC (IGFBP-3 and NKX3.1) and Texas Red (cytokeratin and histone) staining was calculated using average intensity measuring tool of the software. IGFBP-3 and NKX3.1 expression indices were calculated by normalizing intensity of staining to respective internal controls. B. lot of NKX3.1 expression index (cancer vs. normal in individual tissues) versus relative IGFBP-3 expression (cancer versus normal in individual tissues) as determined by confocal quantitative immunomicroscopy. The regression line and correlation coefficient, R, were obtained using Sigmaplot 6.0 software. C. Real-time PCR of IGFBP-3 expression in prostatic tissue of *Nkx3.1* gene targeted mice at 4 and 12 months of age. *Gapdh* expression is shown as a control and was invariant between the different mouse strains. Prostates from wild type and homozygous deletion mice were available from 4 month old animals. For 12 month old animals prostates were also available from heterozygous animals.

Fig 2
NKX3.1 and IGFBP-3 in Tissues

A.



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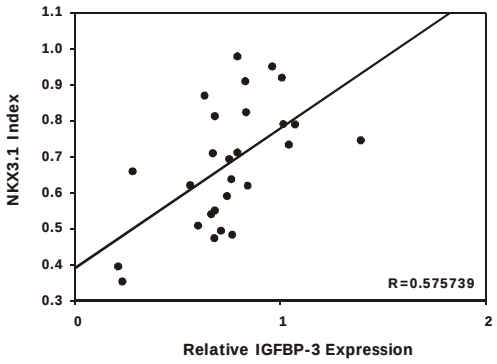

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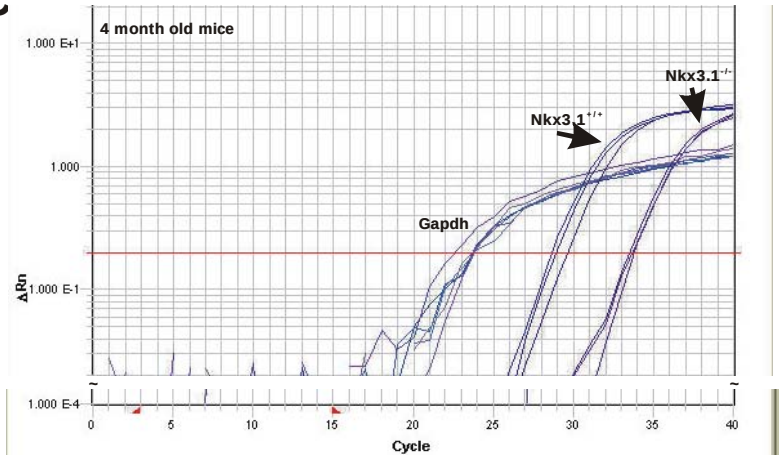
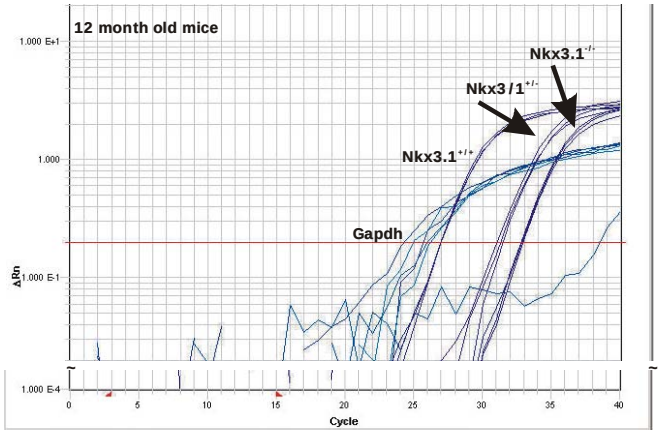




To determine the effect of NKX3.1 expression on cell growth native and derivative PC-3 cells were grown in culture for five days and cell counts were done daily to determine doubling times. The data are shown in Table 1. In each of the three derivative cell lines doubling time was prolonged by the expression of NKX3.1. NKX3.1 expression was verified by western blotting at the termination of each growth curve. NKX3.1 expression reduced growth and prolonged half-life compared to parental or control cultured cells.

Table 1
Effect of NKX3.1 Expression on PC-3 Cell Proliferation

Cell	Doubling Time (hr)	p versus reference
PC-3	25.4 ± 1.9	0.1716
PC-3(pcDNA3.1)	24.3 ± 2.0	Ref
PC-3(NKX3.1) - 1	32.2 ± 5.9	0.0037
PC-3(NKX3.1)-2a	38.8 ± 7.8	0.0002
PC-3(NKX3.1 - 8	30.2 ± 2.6	0.002

To determine if IGFBP-3 mediated the growth effect of NKX3.1 on PC-3 cells we prepared siRNA oligonucleotides and a missense control oligonucleotide as previously described (9). Effective reduction inIGFBP-3 mRNA was seen in PC-3 cell expressing the exogenous vector (Figure 3). Knock down of IGFBP-3 mRNA was achieved at both 24 and 96 hr after addition of the siRNA oligonucleotides to the cultures. This was important because we next determined the effect of the transfection medium alone and the missense oligonucleotides on PC-3 and PC-3(NKX3.1)-1 cell proliferation. PC-3(pcDNA3.1) control transfectants and PC-3(NKX3.1)-1 cells were then subjected to oligonucleotide treatment for control and knock down of IGFBP-3 and cell number was monitored over 96 hr. The results are shown in Table 2. IGFBP-3 siRNA reversed the effect of NKX3.1 expression on growth.

Table 2
Effect of IGFBP-3 Knock Down on Cell Proliferation

Cell Type	Treatment	Doubling Time (hr)	p-value
PC-3(pcDNA3.1)	None	24.9 ± 1.75	reference
PC-3(pcDNA3.1)	Mock	24.9 ± 1.62	0.4730
PC-3(pcDNA3.1)	Missense	23.3 ± 1.70	0.5972
PC-3(NKX3.1)-1	None	32.9 ± 2.30	0.0092
PC-3(NKX3.1)-1	Mock	31.7 ± 2.48	0.0161
PC-3(NKX3.1)-1	Missense	32.0 ± 3.12	0.0219
PC-3(NKX3.1)-1	IGFBP-3 siRNA	24.8 ± 2.56	0.5644

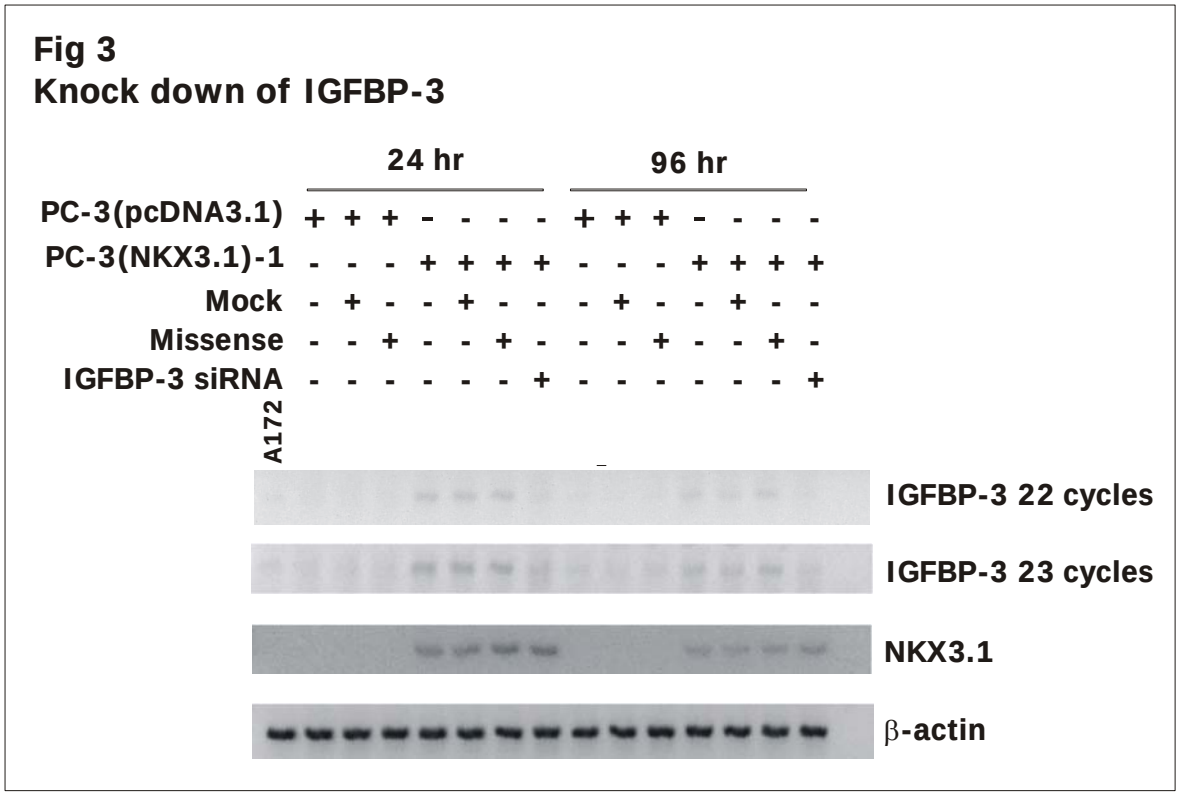


Fig 3. The indicated cell lines were treated with transfection reagent or reagent plus siRNAs as shown and sampled at 24 and 96 hrs. RT-PCR was employed to detect mRNA as indicated on the right. A172 cell mRNA was included as a positive control for IGFBP-3.

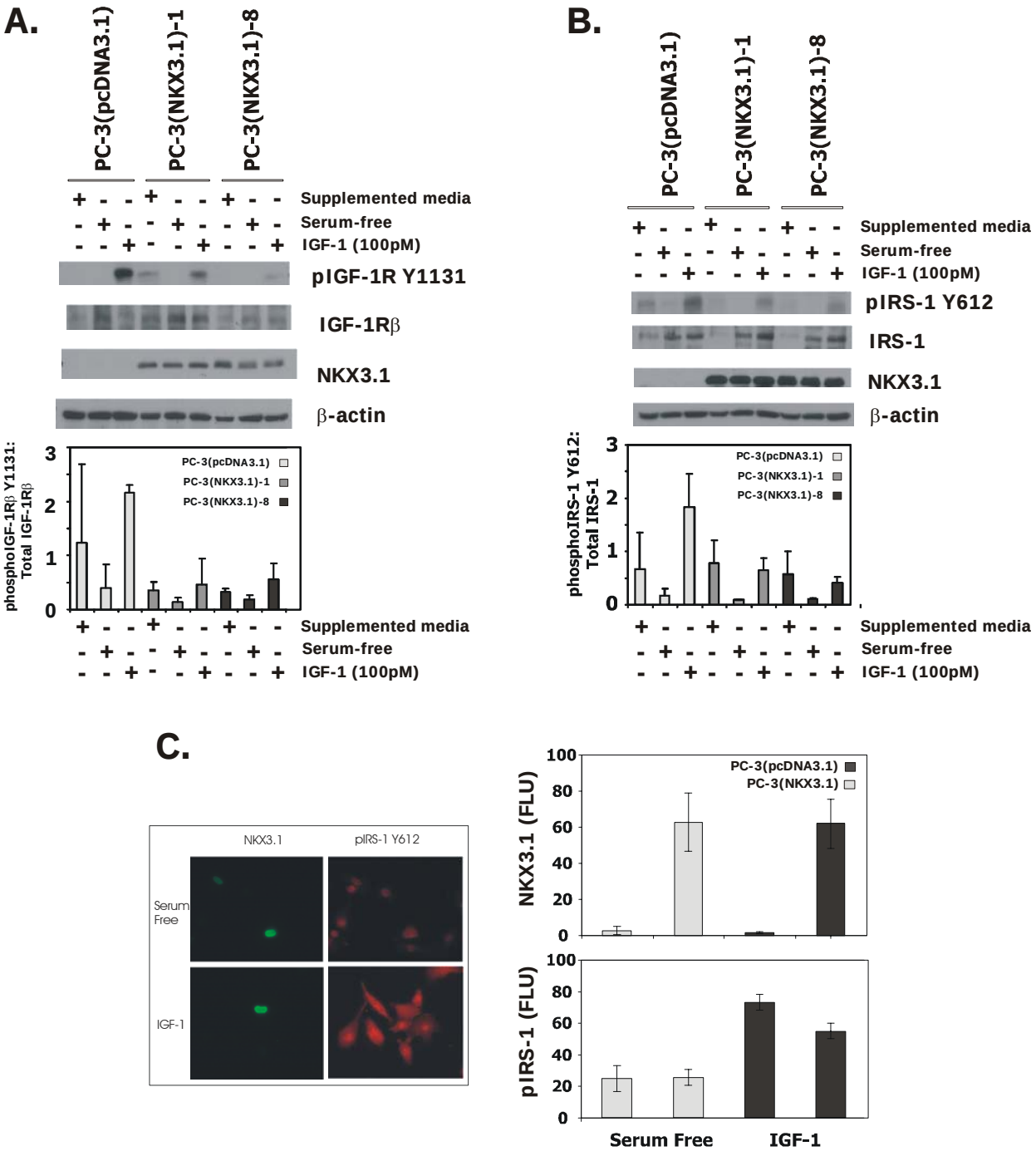
Task 4. Determine the effect of NKX3.1 on IGFR-1 signaling pathways and define the role of IGFBP-3 in the interaction between NKX3.1 and IGFR-1.
Months 19-36.
Assay by western blotting the activation of downstream targets of IGFR-1 and determine the effects of NKX3.1. Targets to be analyzed include:

1. *IGFR-1 phosphorylation will be assayed using monoclonal antibodies specific for insulin receptor that cross-react with IGFR-1 (P-Tyr$^{1135/1136}$).*
2. *IRS-1 is phosphorylated on Y1173 by IGFR-1 and can be assayed by IP-western blotting with antiphosphotyrosine antibody (10) or by Novus antibody to mouse IRS-1(phosphoY1179) that cross reacts with human IRS-1.*
3. *Protein kinase B/AKT is recruited to the cell membrane to PIP3 that results in enzyme activation. We will analyze the effect of the NKX3.1 mutant transgenes and IGFBP-3 siRNA on AKT phosphorylation.*
4. *BAD is a proapoptotic BCL family member that is phosphorylated by AKT on serine 136 and by MAPK cascade at serine 112. We will determine the effects of NKX3.1 and IGFBP-3 siRNA on BAD phosphorylation to examine effects on this component of the intrinsic cell death pathway.*
5. *FOXO1 like other members of the forkhead family of transcription factors is affected by IGFR-1 activation. We will use the antiFOXO1(phosphoserine 256) from Cell Signaling to determine FOXO1 phosphorylation in the NKX3.1 expressing cells before and after IGFBP-3 siRNA treatment.*

To determine the effect of NKX3.1 on signaling downstream from the IGF-1R we analyzed total and phospho-IGF-1R by western blotting in the different stably transfected PC-3 cells. In the presence of NKX3.1 IGF-1R phosphorylation at tyrosine 1131 was diminished (Fig 4A). The western blot in Fig 1A shows diminished phosphorylation of IGF-1R that is quantitated by densitometry in the histogram below. IRS-1 is directly downstream from IGF-1R and is rapidly phosphorylated in response to both insulin and IGF-1. Expression of NKX3.1 diminished IRS-1 phosphorylation in response to IGF-1 in two PC-3 derivative clones examined (Fig 4B). We also examined the effect of NKX3.1 in single cells by two-color immunofluorescent staining for NKX3.1 and phospho-IRS-1 (Fig 4C). The left panel shows an example of a cell stained for NKX3.1 (green nuclei) and phospho-IRS-1 (red) that was quantitated and summarized in the right panel to demonstrate diminished staining for phospho-IRS-1 in the cells that express NKX3.1

Fig 4. NKX3.1 expression inhibits the IGF-1-mediated phosphorylation of the IGF-1R and IRS-1. A. Western blot analysis of IGF-1R phosphorylation in PC-3(control) and PC-3(NKX3.1) cell clones serum-starved and treated with 100pM IGF-1 for 3 minutes. β-Actin was used as a loading control. Densitometric analysis was performed by Scion Imaging software (Scion Corporation) and the graphical representation is based on three or more experiments. B. Western blot analysis of IRS-1 phosphorylation in PC-3(control) and PC-3(NKX3.1) clones serum-starved and treated with 100pM IGF-1 for 3 minutes. C. Immunofluorescent staining of NKX3.1 and pIRS-1 Y612 in PC-3 cells transiently transfected with the NKX3.1 expression vector, serum starved, and treated with 100pM IGF-1 for 3 minutes, immediately followed by fixation. Cells were imaged with an Axiovert 200M deconvolution microscope and fluorescence was quantified using ImageJ (NIH). P-value is <0.002 and was determined by paired t-test using the Prism biostatistics program (Graphpad).

Fig 4
NKX3.1 inhibits IGF-1-mediated phosphorylation of IGF-1R and IRS-1.



We further investigated how NKX3.1 affected signaling downstream from IGF-1R. In two PC-3 clones expressing NKX3.1 phosphorylation of PI-3-kinase in response to IGF-1 was diminished (Fig 5A). In these same cells steady state levels of phospho-AKT were diminished (Fig 5B). To address concerns about clonal variation and selection of cells stably expressing NKX3.1 we also performed transient transfection and showed that NKX3.1 expression in a mass culture diminished the level of phospho-AKT to a similar degree as did transient expression of PTEN (Fig 5C).

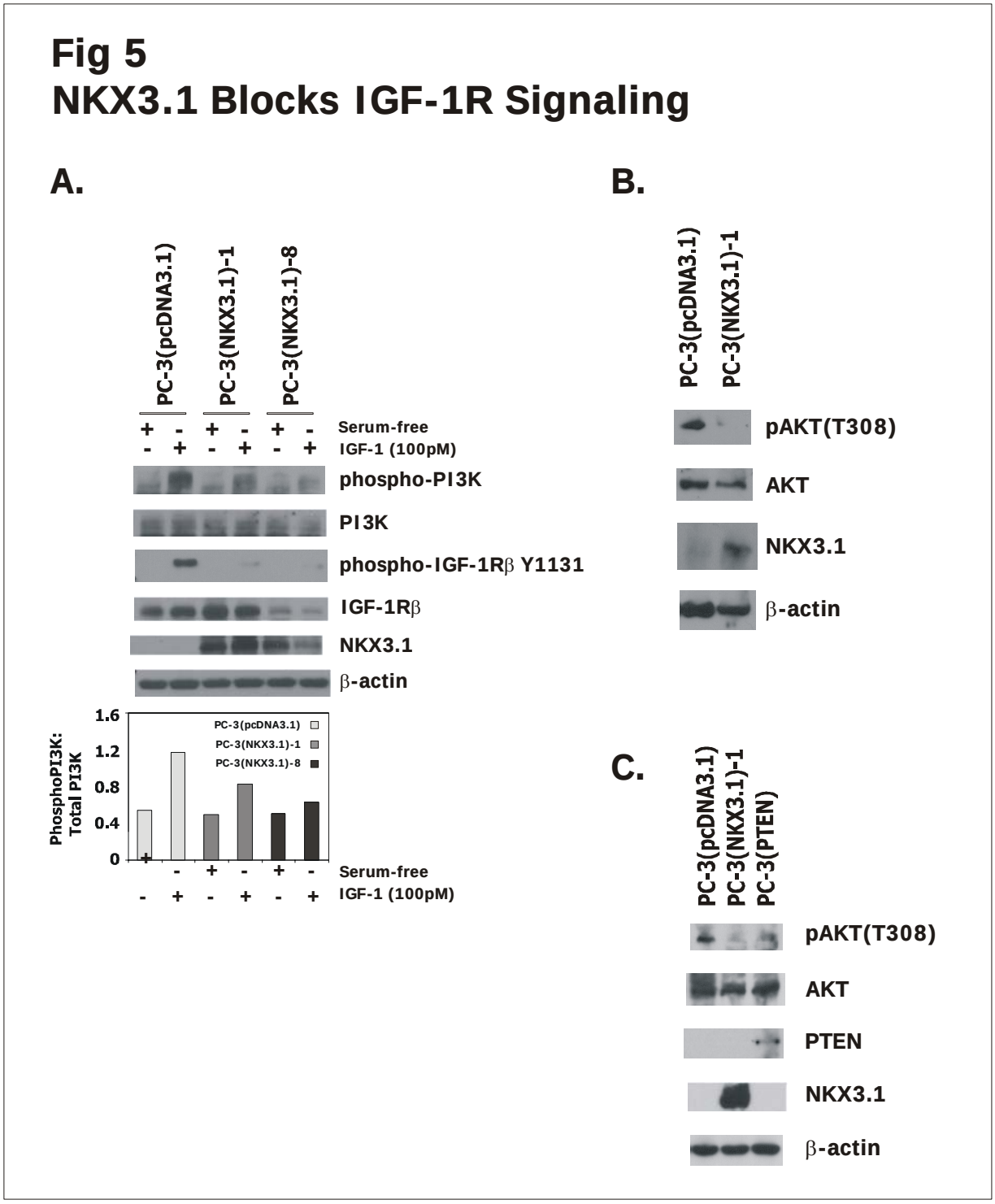
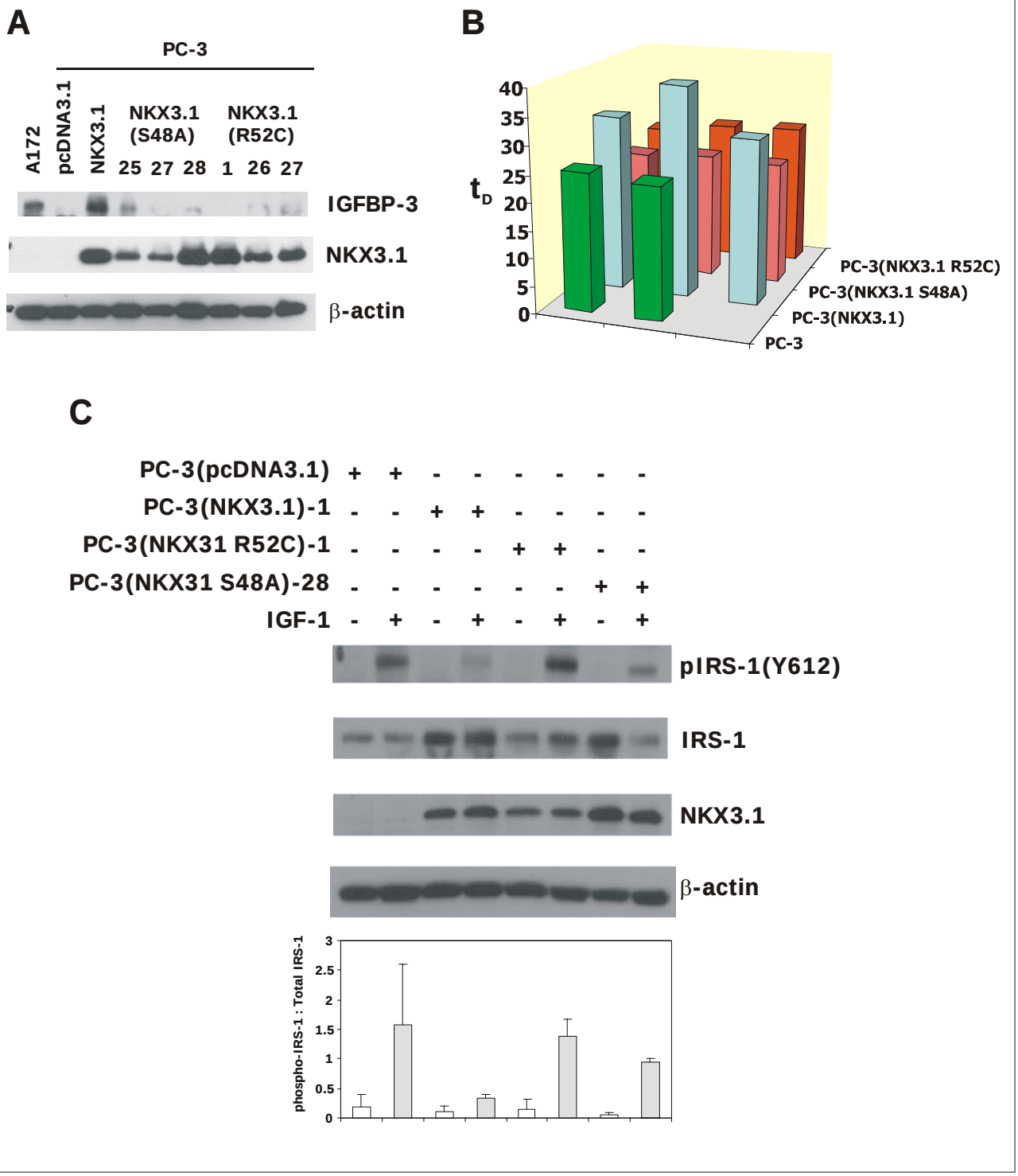


Fig 5 NKX3.1 expression inhibits IGF-1R downstream signaling. A. Western blot analysis of PI-3K phosphorylation in PC-3(pcDNA3.1) and PC-3(NKX3.1) clones serum-starved and treated with 100pM IGF-1 for 3 minutes. β-Actin was used as a loading control. B. Western blot analysis of AKT phosphorylation in cells grown in media containing 10% FBS. C. Western blot analysis of AKT phosphorylation in PC-3 cells transiently transfected with the NKX3.1 and PTEN expression vectors. pcDNA3.1 is the control empty vector and β-Actin was used as a loading control.

Since we have presented preliminary data in the submission of this application indicating that the variant NKX3.1 interacts with serum IGF-1 to increase prostate cancer risk, we asked whether the variant protein NKX3.1(R52C) was attenuated in its ability to activate IGFBP-3 expression. One property of NKX3.1(R52C) is decreased phosphorylation at serine 48 (11). We also constructed the NKX3.1(S48A) serine-to-alanine mutant construct that eliminates the phosphorylation site to test for IGFBP-3 activation. The variant proteins were each expressed in three independent PC-3 clones and examined for induction of IGFBP-3 expression. Compared to wild type NKX3.1 the variant proteins were attenuated in IGFBP-3 induction (Fig 6A). In a 96-hour cell proliferation assay the PC-3 cells expressing variant proteins had doubling times that were not different from control PC-3 cells, but were different from PC-3(NKX3.1) cells (Fig 6B). Consistent with the expectation that variant NKX3.1 proteins would not affect IGF-1R signaling we also showed that the variant proteins did not attenuate IRS-1 phosphorylation compared to wild type NKX3.1 (Fig 6C).

Fig 6. Variant NKX3.1 does not activate IGFBP-3 or affect growth. A. Western blot of cell extracts from individual clones expressing different NKX3.1 constructs as shown. B. Summary of doubling times from individual cell growth curves, each done in triplicate of clones expression NKX3.1 constructs. Expression of the transgene was verified in each case at the completion of the experiment. C. Western blot of cell extracts from the indicated clones grown in serum-free medium or medium + 100pM IGF-1 for 3 minutes. The histogram shows quantitation as described for Fig 4.

Fig 6
Effect of Variant NKX3.1



KEY RESEARCH ACCOMPLISHMENTS:

- NKX3.1 activates IGFBP-3
- IGFBP-3 mediates, in part, growth suppression by NKX3.1
- NKX3.1 affects IGF-1 signaling and activation by IGF1-R by activation of IGFBP-3
- NKX3.1 C154T polymorphism interacts with serum IGF-1 as a prostate cancer risk factor
- *NKX3.1* genotype may magnify prostate cancer risk from circulating IGF-1

REPORTABLE OUTCOMES:

Funding applied for:

CA123086 Risk Factor Interactions in Prostate Cancer. PI – Edward Gelmann

Employment

Erin Muhlbradt, graduate student who participated in this project
Hired as Bioinformatics Research Analyst, Northrup-Grumman. Reston, VA

CONCLUSION: This project is ongoing and is likely to achieve its goals. Our experimental studies have shown that NKX3.1 activates IGFBP-3 expression and that IGFBP-3 mediates to a large degree the growth suppressive effects of NKX3.1 in vitro. The mechanism of this is via interference with the IGF-1 stimulation of the IGF-1R. The variant NKX3.1 coded by the polymorphic locus *NKX3.1* C154T is attenuated in IGFBP-3 activation and growth suppression in vitro. *NKX3.1* C154T is a genetic determinant that sensitizes men to procarcinogenesis in the prostate by circulating IGF-1. This project is likely to validate that finding and establish the mechanistic relationship between a prostate suppressor polymorphism and IGF-1 signaling.

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